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Breakdown of lecithin on aluminum oxide columns*

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SUMMARY

A method is described for the detection of slow breakdown reactions that sometimes occur in chromatographic columns. Hydrolysis of lecithin on aluminum-oxide columns has been demonstrated to occur under conditions normally used in the chromatography of phosphatides. About 1% of lecithin is estimated to be destroyed on alumina columns in 1 hour at 22°. A substantial part of the decomposed lecithin is found as lysolecithin. Lowering the working temperature to 2° markedly reduces the rate of the degradation of lecithin and that of the formation of lysolecithin.

The use of aluminum oxide has become a valuable tool in the fractionation of phosphatides since the work of Hanahan *et al.* (1) and that of Rhodes and Lea (2). Although many authors have probably been concerned about possible hydrolysis of the phosphatides on alumina columns, very little has been done to develop reliable methods for detection of such potential breakdown reactions, and degradation of phosphatides on aluminum oxide has apparently not been previously observed (3).

This paper describes a method for the detection of slow breakdown reactions that sometimes occur during chromatography. It is shown that lecithin is hydrolyzed to lysolecithin on aluminum oxide under conditions normally used in phosphatide chromatography. Observations on eliminating the main part of this destruction are also presented.

MATERIALS AND METHODS

Analytical Methods. The methods for the determination of phosphorus, glycerol, and acyl ester groupings are described elsewhere (4, 5). Paper chromatography of phosphatides was carried out as described by Marinetti *et al.* (6), staining with Rhodamine 6G.

Materials. Lecithin was a synthetic sample of L-α-dipalmitoyl lecithin kindly donated by Fluka AG., Buchs, Switzerland. It was purified by a rapid passage through alkali-free aluminum oxide in chloroform—methanol 8:2. The small amounts of contaminating acidic phosphatides present in the sample were retained in the column. The resulting lecithin preparation assayed correctly for phosphorus, glycerol, and acyl ester groupings. It was also homogeneous on paper chromatography.

The lysolecithin used as a reference standard for paper chromatography was a generous gift from Dr. G. V. Marinetti.

Aluminum oxide (E. Merck AG., Germany), standardized for chromatography according to Brockmann, was used without any pre-treatment. This preparation is referred to as standard aluminum oxide in this report.

The standard aluminum oxide still contained small amounts of soluble alkali, which were removed by exhaustive washing with distilled water and methanol as described by Reichstein *et al.* (7). Reactivation was carried out in a vacuum at 180–185°. The washed and reactivated preparation is referred to as alkali-free aluminum oxide in this report.

Method for the Detection of Lecithin Breakdown on Aluminum Oxide Columns. The method is based on the use of artificially prolonged contact between the two reactants. The same idea was recently used successfully to detect the conversion of one steroid glycoside into another on alumina columns (8).

Lecithin was washed well into the aluminum-oxide column with chloroform and left there long enough for the breakdown reactions to proceed to a measurable extent. Analysis of the reactions was then carried out

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TABLE 1. CHROMATOGRAPHY OF A MIXTURE RESULTING FROM BREAKDOWN OF LECITHIN ON Al_2O_3*

Fr. No.	Eluting Solvent†		Analysis of the Fractions: Phosphorus	Analysis of the Fractions: Acyl Ester	Calculated Composition of the Fractions: Lecithin	Calculated Composition of the Fractions: Lysolecithin	Control by Paper Chromatography†
		ml	*μ atoms*	*μEq*	*μmoles*	*μmoles*	
1	C	20	0.00	2.05	0.00	0.00	
2	C:M (9:1)	10	14.3	28.4	14.30	0.00	PC
3	C:M (8:2)	10	11.3	22.8	11.30	0.00	PC
4	C:M (7:3)	10	4.15	7.11	2.97	1.18	PC + (LPC)
5	C:M (6:4)	10	3.74	4.60	0.86	2.88	(PC) + LPC
6	C:M (5:5)	10	2.06	2.58	0.51	1.55	(PC) + LPC
7	C:M (2:8)	10	1.57	2.07	0.50	1.07	(PC) + LPC
		Sum	37.1	69.6	30.4	6.7	

* Column: 4.0 g alkali-free Al_2O_3; 13 x 42 mm. Load: 49.9 μmoles lecithin incubated in the column for 42 hours at 22° prior to elution. Flow rate during elution 1.5 to 2.0 ml/min.

† Abbreviations used: C = chloroform; M = methanol; PC = lecithin; LPC = lysolecithin.

by eluting the breakdown mixture with chloroform—methanol solutions of increasing methanol concentration. The eluted fractions were subsequently assayed for phosphorus and acyl ester and analyzed by paper chromatography.

Table 1 shows that the breakdown mixture can be divided into fractions:[1] phosphorus-free lipids containing ester groups, unchanged lecithin, lysolecithin, and phosphorus-containing compounds so firmly bound to the column that they are not eluted by chloroform—methanol mixtures.

RESULTS

Breakdown of Lecithin on Standard Aluminum Oxide. Two identical lecithin samples (48.8 μmoles) were chromatographed on 4.0-g standard aluminum oxide columns (13 x 42 mm). One of the samples was eluted immediately, whereas the other was washed into the column with 10 ml of chloroform and left in contact with the adsorbent for 37 hours at 22° before elution was started. Both chromatograms were developed and analyzed as shown in Table 1.

Table 2 shows that 34% of lecithin is degraded after 37 hours of contact with standard aluminum oxide. About 40% of the lecithin destroyed is recovered as lysolecithin.

About 60% of the phosphorus of the lecithin destroyed is so firmly bound to the column that it is not eluted by the chloroform—methanol mixtures used. This firmly bound fraction has been studied only in a preliminary manner, using material obtained from incubation experiments similar to that in Table 2. Partial elution of this fraction was obtained with large volumes of chloroform—alcohol—water mixtures, and the eluted material was found to contain additional amounts of lysolecithin and smaller amounts of unidentified acidic phosphatides. Only about 70% of the lysolecithin formed during the incubation was eluted from our columns by the dry chloroform—methanol mixtures used. Direct analysis of the rest of the firmly bound fraction is not yet possible as the elution characteristics of phosphatidic and lysophosphatidic acids are unknown. Indirectly, however, some insight into the composition of this fraction may be gained from the fact that the phosphorus-free acyl esters from the incubation experiments contained only negligible amounts of glycerol (molar ratio of acyl ester to glycerol, about 20). This suggests that cleavage between the glycerol and the phosphoric acid moieties of lecithin is probably insignificant.

Assuming that the destruction of lecithin is a first-order reaction, the results shown in Table 2 mean

[1] The exact determination of lecithin and lysolecithin is possible even in the incompletely resolved fractions by simultaneous assays of phosphorus and acyl ester, because paper chromatography shows that no other lipids are present in these mixtures.

TABLE 2. RESULTS OF CHROMATOGRAMS OF LECITHIN SAMPLES (48.8 μMOLES) ON STANDARD ALUMINUM OXIDE

Fraction	Chromatogram Eluted Immediately	Chromatogram Eluted After 37 Hours of Incubation
	μmoles	*μmoles*
Phosphorus-free acyl ester	0.63	1.35
Lecithin	46.4	30.6
Lysolecithin	0.1	7.5
Phosphorus not eluted	2.3	10.6

TABLE 3. RESULTS OF CHROMATOGRAMS OF LECITHIN SAMPLES (49.9 μMOLES) ON STANDARD AND ALKALI-FREE Al_2O_3 AFTER 42 HOURS OF INCUBATION

Fraction	Standard Al_2O_3	Alkali-Free Al_2O_3
	μmoles	*μmoles*
Phosphorus-free acyl ester	1.34	2.05
Lecithin	30.2	30.4
Lysolecithin	11.6	6.7
Phosphorus not eluted	8.1	12.8

TABLE 4. RESULTS OF CHROMATOGRAMS OF LECITHIN SAMPLES (51.2 μMOLES) ON ALKALI-FREE Al_2O_3 AFTER 45 HOURS OF INCUBATION AT DIFFERENT TEMPERATURES

Fraction	Incubation at 22°	Incubation* at 2°
	μmoles	*μmoles*
Phosphorus-free acyl ester	3.7	1.4
Lecithin	27.9	43.6
Lysolecithin	8.2	1.4
Phosphorus not eluted	15.1	6.2

* Both columns were developed at 22°.

that about 1.1% of lecithin is decomposed in 1 hour at 22° on standard alumina columns.

Breakdown of Lecithin on Alkali-free Aluminum Oxide. A comparison of the breakdown of lecithin on standard and alkali-free aluminum oxide was made by simultaneously incubating two identical samples of lecithin (49.9 μmoles) for 42 hours at 22° on 4.0-g columns of the adsorbents.

Table 3 shows that the alkali-free aluminum oxide causes as great destruction of lecithin as does the standard alumina. The various breakdown products are also formed in similar amounts on the two adsorbents.

Effect of Temperature on Breakdown. Lecithin breakdown on alkali-free aluminum oxide at 22° was compared to that occurring at 2° by simultaneously incubating identical samples of lecithin (51.2 μmoles) for 45 hours at these temperatures.

Table 4 shows that at 2° the rate of lecithin breakdown, as well as the rate of formation of lysolecithin and of phosphorus compounds not eluted, is markedly reduced.

DISCUSSION

The observations presented in this report suggest that, in many previous studies in which natural lipid mixtures have been fractionated by chromatography on aluminum oxide, a part of the lysolecithin found may have been formed from lecithin.

As we have also observed similar degradation of the inositides on aluminum oxide, it seems quite probable that the destruction of lecithin reflects a general capacity of aluminum oxide to cause hydrolysis of alkali-labile lipids. We think that the incubation technique described above will prove useful in establishing the validity of this theory.

We think that the use of low working temperatures in aluminum-oxide chromatography of lipids is the best way to eliminate the observed destructive reactions. In our experience, aluminum oxide retains its fractionating power at low temperatures.

We have further observed that on silicic-acid columns, the dipalmitoyl lecithin is not degraded at all, whereas the vinyl-ether linkages of highly purified choline plasmalogens are hydrolyzed considerably. If, conversely, alumina causes general deacylation of phosphatides but does not cause hydrolysis of their aldehyde groupings, it may prove to be more useful for fractionation of phosphatides in some cases.

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